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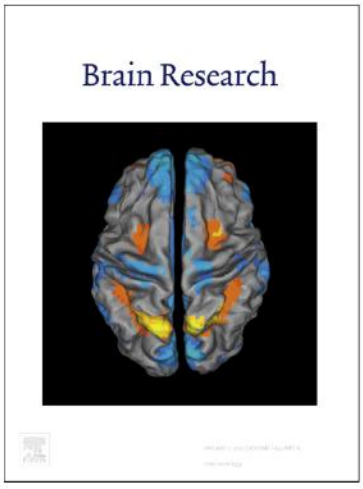

Research report

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Postnatal genistein administration selectively abolishes sexual dimorphism in specific hypothalamic dopaminergic system in mice.

Giovanna Ponti[2,3 *], Alice Farinetti[1,2], Marilena Marraudino[1,2], GianCarlo Panzica[1,2], Stefano Gotti[1,2]

[1] Department of Neuroscience "Rita Levi-Montalcini" Via Cherasco 15, 10126-Turin (TO)

[2] Neuroscience Institute Cavalieri Ottolenghi (NICO), Regione Gonzole, 10-10043 Orbassano (TO), University of Turin, Turin, Italy.

[3] Department of Veterinary Sciences, Largo Braccini 2, 10095 Grugliasco (TO), University of Turin, Turin, Italy.

* corresponding author:
Giovanna Ponti
Department of Veterinary Sciences,
Largo Braccini 2, 10095 Grugliasco (TO),
University of Turin, Turin, Italy.
tel: +39-011-6709144
fax: +39-011-6709138
e-mail: giovanna.ponti@unito.it

Highlights

- Hypothalamic dopaminergic subpopulations are affected by neonatal EDC treatment
- GEN and E_2 alter the development of different nuclei in both sexes
- Mesencephalic derived dopaminergic neurons are not sensible to the tested EDC

Abstract

As demonstrated in previous studies, early postnatal genistein (GEN) administration to mice pups of both sexes, at doses similar to that of infant soy-based formulas, may affect the development of some steroid-sensitive neuronal circuits (i.e. nitrergic and vasopressinergic systems), causing irreversible alterations in adults. Here, we investigated the hypothalamic and mesencephalic dopaminergic system (identified with tyrosine hydroxylase immunohistochemistry). GEN administration (50 mg/kg) to mice of both sexes during the first week of postnatal life specifically affected tyrosine hydroxylase immunohistochemistry in the hypothalamic subpopulation of neurons, abolishing their sexual dimorphism. On the contrary, we did not observe any effects in the mesencephalic groups. Due to the large involvement of dopamine in circuits controlling rodent sexual behavior and food intake, these results clearly indicate that the early postnatal administration of GEN may irreversibly alter the control of reproduction, of energetic metabolism, and other behaviors. These results suggest the need for a careful evaluation of the use of soy products in both human and animal newborns.

Keywords

phytoestrogens, endocrine disruptor compounds, sexual dimorphism, hypothalamus, dopaminergic system, midbrain neurons

Abbreviations

AVPV anteroventral periventricular nucleus

ARC arcuate nucleus

BPA Bisphenol A

E_2 17-β-estradiol

ERα estrogen receptor α

EDC endocrine disrupting chemical

GEN genistein

GnRH gonadotrophin releasing hormone

GHRH growth hormone releasing hormone

MSH melanostimulating hormone

PAG periacqueductural grey

PBS phosphate saline buffer

Pe periventricular part of the paraventricular nucleus

PVN hypothalamic paraventricular nucleus

ROI region of interest

SN substantia nigra

TH tyrosine hydroxylase

VTA ventral tegmental area

ZI zona incerta thalamus

1. Introduction

Tyrosine hydroxylase (TH) is the key enzyme for catecholamine synthesis. Therefore, TH immunoreactivity (TH^+) is the most diffuse marker for adrenergic, noradrenergic and dopaminergic neurons. Since adrenergic and noradrenergic cell bodies are restricted to the hindbrain, all TH^+ cell bodies in other regions of the brain are considered to belong to dopaminergic neurons. They constitute a largely diffused system involved in the modulation of many behaviors both directly and through their interactions with other neuronal systems (Chu and Etgen 1997, Hull and Dominguez 2006).

The mesencephalic and prosencephalic TH+ dopaminergic neurons have been classified, in rats (Dahlstroem and Fuxe 1964) and in mice (Zaborszky and Vadasz 2001), into 8 groups (from A8 to A15). They belong to two main anatomical groups originating from two different embryonic progenitors: mesencephalic neurons arise from midbrain mesomeres of the floor plate of the diencephalon while diencephalic ones derive from hypothalamic prosomeres located in the alar plate of the diencephalon (Puelles and Rubenstein 2015). The first group originates from three midbrain clusters: A10, A9 and A8. TH^+ neurons of the A10 group (ventral tegmental area, VTA) are part of the motivation and reward circuit (Wise and Rompre 1989) and are involved in stress response, anxiety disorders (Baskerville and Douglas 2010), and emotional processes of learning and memory (Kvetnansky et al., 2009). The TH+ neurons of the A8-A9 groups (Ventromedial substantia nigra, SN) modulate the motor system and motivated behavior (Sotomayor-Zárate et al. 2014).

Diencephalic TH+ neurons (A11-A12-A13-A14-A15 groups) encompass hypothalamic nuclei implicated in the control of energetic metabolism, stress and fertility. The A12 group, in the ARC, is involved in the control of energetic metabolism and fertility (De Bond and Smith 2014, Iijima et al. 2015, Iwata et al. 2016, Marraudino et al. 2018). The A13 group belongs to the zona incerta, where interneurons controlling GnRH release are present (MacKenzie et al., 1988). The A14 group is formed by a more rostral part located in the anteroventral periventricular nucleus (AVPV) and a more caudal part, consisting of a group of neurons near the ventricular wall (periventricular, Pe) along with others that lie more deeply in the parenchyma of the hypothalamic paraventricular nucleus (PVN) (Baker et al. 1983, Vastagh and Liposits 2017), a key relay center controlling stress, reproduction and energetic metabolism (Argiolas and Melis 2004, Herman and Tasker 2016, Kageyama and Suda 2009, Lozic et al. 2018). In the AVPV, approximately half of kisspeptin neurons regulating GnRH neurons are also TH+ (Clarkson and Herbison 2011), although the functional significance of TH in those neurons is still unknown (Stephens et al. 2017). As for many areas of the vertebrate brain, several dopaminergic cell groups are sexually dimorphic: AVPV and ARC display more TH+ neurons in females than in males (Balan et al. 2000, Simerly 1989). Brain sex differences may be caused by different mechanisms (Becú-Villalobos et al. 1997, Lenz et al.,

2012, Negri-Cesi et al. 2004, Orikasa and Sakuma 2010, Panzica and Melcangi 2016): the presence of sex chromosomes (i.e. XX or XY), the presence of specific genes (i.e. the Sry gene) inducing gonads' differentiation (and therefore enabling sex specific endocrine signaling), or epigenetic events (Nugent et al. 2015) during developmental critical periods. For instance, the presence of sex chromosomes is responsible for the sex dimorphism of the mesencephalic population of TH+ cells. In fact, the use of the "four core genotypes" (FCG) mouse model (Arnold 2009, Arnold and Chen 2009, Carruth et al., 2002) demonstrated that the Sry gene is not implicated in the induction of the sex dimorphism of the mesencephalic population of TH+ cells; therefore this dimorphism is due to the other genes located on the Y chromosome. On the contrary, ERα-KO males show a feminization of the TH+ population in the AVPV, demonstrating that the perinatal hormonal environment deeply influences the differentiation of this cell group (Simerly et al. 1997).

The dimorphisms of neural circuits may be altered by the exposure to some molecules defined as endocrine disrupting chemicals (EDCs) (Frye et al. 2012, Gore and Dickerson 2012, Street et al. 2018). Many EDCs act as xenoestrogens; therefore, given the relevance of sex hormones for the differentiation of some TH+ systems, several studies have addressed the sensitivity of these neurons to EDCs. For example, early administration of bisphenol A (BPA) alters TH expression in AVPV in rats (McCaffrey et al. 2013). Phytoestrogens, as genistein (GEN), are a class of EDCs, which raise a special concern due to their abundance in soy-based food including formula for babies and nutritional supplements for livestock. In fact, depending on the age, the effects of GEN exposure may be very different. GEN administration in adults may lead to beneficial effects (Azcoitia et al. 2006, Barnes 1998, Branca and Lorenzetti 2005). On the other hand, at critical stages during perinatal development, it may cause anatomical changes in specific neuronal circuits (Losa et al. 2011, Lund et al. 2001, Patisaul et al., 2006, Ponti et al. 2017, Rodriguez-Gomez et al. 2014, Viglietti-Panzica et al., 2007), leading to persistent detrimental effects (Patisaul and Jefferson 2010). Indeed, in many animal models, early GEN exposure affects aggressive (Rodriguez-Gomez et al. 2014, Wisniewski et al. 2005), sexual (Sullivan et al., 2011, Viglietti-Panzicaet al., 2007) and anxiety-related behaviors (Lephart et al. 2004, Patisaul et al. 2012, Rodriguez-Gomez et al. 2014). Moreover, it impairs fertility (Losa et al. 2011, Lund et al. 2001), and energetic metabolism (Strakovsky et al. 2014; Marraudino et al., 2019). GEN, as other EDCs (Yang, et al. 2015), is able to induce both estrogenic and anti-estrogenic effects (Ponti et al. 2017), as well as acting independently from estrogen receptors' binding (Chacko et al. 2005, Polkowski and Mazurek 2000, Kim et al. 2004).

In our previous studies, we have shown that GEN administration, during prenatal life (Rodriguez-Gomez et al. 2014) or in the first postnatal week, alters nitrergic and vasopressinergic circuits in mice (Ponti et al. 2017). Here we studied the effect of neonatal GEN administration on the different TH^+ neuronal groups.

We have also included a group of mice treated with E_2 in order to assess if GEN is acting as a xenoestrogen in part of or in the whole TH system we analyzed.

2. Results

2.1 Physiological and body parameters

As expected, body weight (BW) at birth was not different among the groups (females OIL 1.83+/-0.04, E2 1.79+/-0.03, GEN 1.67+/-0.04; males OIL 1.74+/-0.02, E2 1.90+/-0.05; GEN 1.77+/- 0.03) whereas the BW was significantly different among sexes at PND60 ($p < 0.001$). At sacrifice, no significant differences were observed in male BW (OIL: 33.74+/-0.70g; E_2 34.79+/-1.15g; GEN 36.12+/-0.77g; $p = 0.70$), as well as in females (OIL: 26.08+/-0.64 g; E_2 27.33+/-0.63g; GEN 27.79+/-1.03g; $p=0.38$). However, the body weight gain (BWG), was increased in females (Females OIL: 14.28+/-0.66; E_2 15.46+/-0.39; GEN 17.08+/-0.82; Males OIL: 19.53+/-0.41; E_2 18.02+/-0.32; GEN 19.82+/-0.55; $p=0.02$ Fig 1A),

GEN treatment had an impact on females' estrous cycle. In fact, while all the control females had regular cycles, only 40% +/-3.33 of E_2-treated females and none of GEN-treated females had regular cycles. In particular, we observed a high variability among the days in each phase, e.g. percentage of days in estrous (OIL 23.02+/-5%; E_2 33.33+/-12% GEN 32.32+/-16.26%)

Uterus, testis and prostate weight were measured at sacrifice. E_2 and GEN treatment slightly increased the ratio of uterus vs. BW but the difference was not statistically significant. By contrast, we observed a statistically significant difference in the ratio of prostate vs. BW between the three experimental groups (one-way ANOVA: $F=3.985$, $p=0.035$). E_2 treatment had no effect, whereas GEN treated males presented a lower ratio prostate/BW in comparison with the controls (OIL: 1.02+/-0.06; E_2 0.99+/-0.06; GEN 0.83+/-0.04Tukey post hoc test, OIL vs. GEN, $p= 0.050$; Fig. 1B). Furthermore, both E_2 and GEN treatment slightly decreased the ratio of testis/BW, although this difference was only close to statistical significance (OIL: 0.75+/-0.04; E_2 0.66+/-0.07; GEN 0.68+/-0.04 one-way ANOVA: $F=0.708$, $p=0.505$; Fig. 1C).

Puberty, assessed by vaginal opening, occurred between PND25 and PND32 with no significant differences among experimental groups.

2.2 Immunohistochemical results

The immunohistochemical reaction was carried out on brain sections from Bregma 2.68 to -4.72. As expected, in each specimen TH^+ cells and fibers were observed in all regions described in the literature, and the control animals, treated with sesame oil, displayed a staining similar to that previously described (Baker et al. 1983, Zaborszky and Vadasz 2001).

In all experimental groups, clearly labeled cell bodies were present in AVPV, dPVN, vPVN, ARC, ZI, PAG, SN, and VTA; in this last nucleus cell bodies were surrounded by a dense meshwork of labeled fibers. All the data collected with the quantitative analysis are summarized in the Tab.1 and the values of the density of TH^+ structures are expressed as Mean ± Standard Error.

2.2.1 ALAR PLATE DERIVED DOPAMINERGIC GROUPS (A15 –A12 groups).

2.2.1.1 A15: Anteroventral periventricular nucleus (AVPV)

A group of TH^+ cells that span from Bregma 0.62 to Bregma 0.26 (corresponding to A15 group) were measured in a single representative section for each animal. This group corresponds to the Anteroventral periventricular nucleus (AVPV). Here, we observed round cell bodies displaying a clear immunoreactivity in every experimental group (Fig. 2C-H). As previously described (Scott et al. 2015), control females displayed a higher TH^+ cell density than males in the AVPV (Tab. 1; Fig. 2B, C,F). The statistical analysis showed significant effects of both sexes ($F_{(1,24)} = 36.553$, $p<0.0001$) and the interaction between sex and treatment ($F_{(1,24)} = 6.469$, $p=0.007$), over the density of TH^+ cells in AVPV (Fig. 2B). In every group of males the density of labeled cells was lower than in the groups of females, but the statistical analysis confirmed a significant sexual dimorphism only in OIL and E_2 groups (Tukey post hoc test: Female OIL vs Male OIL: $p<0.0001$; Female E_2 vs Male E_2: $p=0.003$; Fig. 2; Tab. 1). On the other hand, GEN treatment seems to abolish sexual dimorphism in TH^+ cell density. In fact, while GEN treatment tended to increase the TH^+ cell density in males (Tab. 1; Fig. 2F, H), it caused a significant decrease in females compared to controls (Tukey post hoc test: Female OIL vs. Female GEN: $p=0.027$ Fig. 2C,E; Tab. 1).

2.2.1.2 A14: Paraventricular nucleus (PVN)

We analyzed three sections at different rostro-caudal levels of the A14 group corresponding to the *Paraventricular nucleus (PVN).* We observed darkly stained cell bodies in a thin meshwork of lightly stained fibers, and the TH^+ cell density was decreasing from rostral to caudal levels (Tab. 1). At each examined level, the density of TH^+ cells was similar in males and females of the control groups; moreover, neither E_2 nor GEN treatment significantly changed this feature (Tab. 1). Since the PVN encompasses different subnuclei, we divided the whole area into a ventral part (vPVN) and a dorsal one (dPVN; Suppl. Figure). In both regions, the TH^+ cell density displayed a decreasing gradient along the rostrocaudal axis (Tab. 1). The statistical analysis did not reveal differences among the experimental groups in either the dPVN or in vPVN in any of the considered levels (Tab. 1).

2.2.1.3 A13: Zona incerta of the thalamus (ZI)

For each hemisphere, we evaluated the cell density of the *A13* group that corresponds to the *Zona incerta of the thalamus (ZI)* in a coronal section corresponding to -1.34 mm from Bregma (Fig. 3). The statistical analysis revealed significant effects of sex and the interaction of sex and treatment, but not the treatment, on the distribution of TH^+ cell (Two way ANOVA: Sex: $F_{(1,23)} = 6.619$, $p=0.019$; Sex*Treatment: $F_{(1,23)} = 4.516$, $p=0.026$). In fact, in control groups, TH^+ cell density was lower in males compared to females (effect of sex, Tukey post hoc test: $p=0.028$; Tab. 1; Fig. 3). This dimorphism was abolished by both GEN and E_2 treatments, but in a different way in males and females (effect of the interaction of sex and treatment: Tab. 1; Fig. 2). In fact, although, treatments did not change significantly the TH+ cell density (Tab. 1; Fig. 3), they had a sex dimorphic effect: in females, they tended to decrease the TH+ cell density in comparison with the control group (Fig. 3), while in males they induced a slight increase compared to the control group.

2.2.1.4 A12: Arcuate nucleus (ARC)

For the *A12* group, corresponding to the *Arcuate nucleus (ARC)* we analyzed the number of TH^+ cells at two different rostro-caudal levels (Bregma -1.46 and -1.70) (Suppl. Figure). Since both the TH^+ cell density and the statistical analysis (two-way ANOVA for repeated measures) were different in the two rostrocaudal levels, we analyzed them separately (Tab. 1) and report the results for the anterior and posterior parts of the ARC.

In the anterior part of the ARC, the statistical analysis (two-way ANOVA with sex and treatments as independent variables) revealed significant effects for sex, treatment and for the interaction between them (Sex: $_{(1,24)} = 30.710$, $p < 0.001$, Treatment: $F_{(1,24)} = 4.690$, $p=0.022$; Sex*Treatment: $F_{(1,24)} = 6.472$, $p=0.007$). At this level, control females displayed a higher TH^+ cell density in comparison with males, (Tukey post-hoc test: $p=0.005$; Fig. 4). The distribution of TH^+ cells was different in the experimental groups also in the posterior part of ARC. The two-way ANOVA reported significant differences for sex, treatment, and their interaction (Sex: $F_{(1,23)} = 17.043$, $p = 0.001$, Treatment: $F_{(1,23)} = 8.370$, $p=0.003$; Sex*Treatment: $F_{(1,23)} = 12.133$, $p < 0.001$). However, in the control group there was no significant difference between sexes, even if the number of cells were higher in females.

The E_2 treatment induced an increase in the TH^+ cell density in males at both levels (Tukey post-hoc test: ARC Ant: Male OIL vs Male E_2, $p=0.010$, ARC Post: Male OIL vs Male E_2, $p= 0.008$, Tab. 1; Fig. 4), depicting a disappearance of the sex differences observed in the control group. GEN treatment had no effect in males at both levels of ARC (Tab. 1), while it increased the TH^+ cell density in females at the posterior level (Tukey post-hoc test: Female OIL vs Female GEN, $p=0.05$; Fig. 4).

2.2.2 FLOOR PLATE DERIVED DOPAMINERGIC GROUPS (MESENCEPHALIC NUCLEI)

2.2.2.1 A9: Substantia nigra (SN)

Clearly labeled cells, corresponding to the medial A9 cluster (Fu et al. 2012, Zeiss 2005), were present in the pars compacta of the *Substantia nigra (SN)*, while only few fibers were present in the *pars reticulata*. We counted the cell density at two rostrocaudal levels (anterior and posterior SN, 3.08 and -3.52 mm from Bregma). The ROI covered most of the *pars compacta* (Suppl. Figure). Many darkly labeled TH^+ cell bodies were evident despite the high number of fibers in the area. The statistical analysis showed no significant differences in the distribution of TH^+ structures between experimental groups in the whole SN or in each of the different rostro-caudal levels.

2.2.2.2 A10: Ventral tegmental area (VTA)

TH^+ cells of the A10 group are located in the *Ventral tegmental area (VTA)*. Cell bodies here are intermingled in a dense meshwork of fibers, preventing an easy cell counting. In this region, we therefore measured the percentage (fractional area) of the ROI covered by immunoreactive structures (cell bodies and processes) in a coronal section corresponding to -3.52 mm from Bregma (Suppl. Figure), located in the parabrachial pigmented nucleus (Fu et al. 2012). The statistical analysis showed no sexual dimorphism in control animals, nor a significant effect of either GEN or E_2 treatment in the distribution of the TH^+ structures (Tab. 1).

2.2.2.3 A10: Periacqueductal grey (PAG)

A small group of A10 TH^+ cells is also clustered in the *Periacqueductal grey (PAG)* (Vanderhorst, Gustafsson, and Ulfhake 2005, Yang, Liu, et al. 2015). TH+ cells were counted in the dorsolateral column in a coronal section corresponding to – 3.52 mm from Bregma. No significant differences were observed among experimental groups (Tab. 1).

3. Discussion

We analyzed here the effects of early postnatal exposure to GEN at a dose comparable to that of infant formulas (Cimafranca et al. 2010) on the hypothalamic and mesencephalic TH+ system. The study is a follow-up of our previous investigation showing that this administration paradigm altered the normal development of estrogen sensitive brain circuits leading to widespread permanent effects in $nNOS^+$ and AVP+ neuronal circuits (Ponti et al. 2017).

The two groups of neuronal progenitors of dopaminergic neurons reside in the alar plate and in the floor plate of the diencephalon (Marin et al. 2005). They both proliferate between embryonic days 8.5 (E8.5) to E13.5 (Kee et al. 2017) and, at the time of our treatment, they undergo programmed cell death followed by

an extensive neuritic outgrowth and stabilization of synaptic connections, acquiring their physiological properties (Luo and Huang 2016).

As previously reported (see Introduction), several groups of the TH+ system are sexually dimorphic, in particular, in both mesencephalic and diencephalic groups in rats (Beyer, Pilgrim, and Reisert 1991, Simerly 1989) and in C57BL/6 mice (Dewing et al. 2006). As demonstrated by experiments performed on the FCG model, the origin of this dimorphism may be linked to an effect of the Sry gene or to other genes (Dewing et al. 2006, Carruth et al., 2002, Panzica and Melcangi 2016). Interestingly, in our experiment, performed with CD1 mice, we did not observe sexual dimorphism in the dorsal part of the SN (pars compacta), in the PAG, or in the VTA. Similar results were obtained in C3H/He mice (Tanida et al. 2009). It is therefore possible that the sex differences of the mesencephalic TH+ system in mice are strain specific (Vanderhorst et al., 2005).

Similarly, we did not observe any differences for the TH+ cells in PAG of E_2- or GEN-treated animals, despite the fact that those cells express ERα (Vanderhorst et al., 2005). It is interesting to note that midbrain TH^+ neurons are sensitive to BPA, phthalate, or dioxin perinatal exposure (Tanida et al. 2009), whereas GEN or E_2 do not affect these cell populations (present study). This is probably due to different chemical pathways involved, the thyroid hormone receptor for BPA, phthalate or dioxin (Moriyama et al. 2018), and the estrogen receptor for E_2 or GEN.

On the other hand, we confirm here the presence of sex dimorphism of the alar plate derived dopaminergic groups, with a higher TH^+ cell density in females than in males (Patisaul and Polston 2008, Simerly et al. 1997, Simerly 1989, Simerly et al., 1985). Previous studies demonstrated that, for example in the AVPV, some EDCs alter the expression of the TH system as well as that of ERα. In particular, exposure to BPA or GEN induced a demasculinization of the rat TH system, i.e. an increase in cell density (Patisaul et al., 2006). In addition, GEN alters other hypothalamic circuits, including the nNOS and the AVP systems (Ponti et al. 2017).

Several studies have demonstrated that many of the hypothalamic sexually dimorphic structures are determined by gonadal hormones' signaling during development (for a recent review see (Panzica and Melcangi 2016), for this reason, they are also sensitive to EDCs with xenoestrogenic activity (Panzica et al. 2007) . The action of post-natal GEN on rodents' brain only partially support this idea. In fact, it is true that the neonatal exposure to GEN may affect many hypothalamic structures such as the AVPV (Patisaulet al., 2006), medial preoptic area, PVN and ARC (Ponti et al. 2017), but this treatment targets specific cell populations. In fact, while both nNOS and AVP neurons in the PVN are affected by GEN administration, this treatment did not alter TH^+ cell density. On the other hand, other hypothalamic TH+ neurons (i.e. AVPV and ARC) were affected by the treatment. TH^+ cell density in ARC was higher in females. E_2 treatment was able to increase TH^+ cell density in ARC resulting in a feminization effect in males while

no effect was observed in GEN treated animals. Since TH+ neurons in AVPV are implicated in the onset of puberty and estrous cycle regulation (Stephens et al. 2017), it is possible to speculate that this effect is involved in the advanced menarche age (D'Aloisio et al. 2013) and in the irregularities in the menstrual cycle observed in women that have been fed with soy based formulas (Upson et al. 2016). On the other hand, the changes in TH+ neurons density in the ARC, may be related with an increased risk of developing metabolic disorders during adult age (Strakovsky et al. 2014). In fact, most of them co-express GHRH (about one third of GHRH neurons in the ARC co-express TH+, Bouyer et al., 2007). In rats, most of GHRH neurons in ARC display ERα receptor while only few of them express ERβ (Shimizu et al., 2005) and are therefore sensitive to adult E_2 treatment (Paison et al.,1992). ERα is precociously expressed in the ARC during the postnatal period in rodents (Cao and Patisaul 2011). On the contrary, ERβ is absent in the ARC during the early postnatal period (PND 3-7 in rats; Pérez et al., 2003). Since GEN has higher affinity for ERβ than ERα (Kuiper et al., 1998), the lack of ERβ in ARC TH+ cells during the early postnatal period may explain why we saw an effect of E_2 but not of GEN in this region.

The risk of developing metabolic disorders during adult age may be related also to the effect on ZI. In fact, this area is implicated in binge eating (Zhang and van den Pol 2017), and alteration at this level may be involved in developing obesity. However, we cannot exclude that other responses related to TH+ cells in ZI may be affected by this treatment, namely locomotion (Sharma et al. 2018) and reward-related behavior (Li et al., 2014). However, in humans, epidemiological data on these adverse effects of GEN are still incomplete (Westmark 2016).

Present results show that the effect of GEN was not the same in both sexes, confirming previous findings of our and other laboratories (Rebuli and Patisaul 2016, Ponti et al. 2017), and highlighting the importance of performing experiments on both sexes (Lee 2018).

As expected (Clarkson et al. 2014, Clarkson et al. 2012), control animals displayed a lower density of TH^+ cells in AVPV of males compared to females. Indeed, we observed that GEN treatment, abolished sexual dimorphism in AVPV by decreasing the TH^+ cell density in females and increasing it in males. This effect may be even more evident since we analyzed the animals in diestrus when TH expression in AVPV is lower than in proestrus (Vastagh and Liposits 2017). Interestingly, Patisaul and coworkers (Patisaul et al., 2006) observed a similar increase in the TH^+ cell density in AVPV of GEN treated male rats but not the opposite effect that we observed in female mice. It is possible that a single oral administration/day of 50mg/kg/day of GEN was more effective to masculinize the TH^+ cell density in females, than the same dose divided in two subcutaneous injections, or that the effect of the treatment is not yet evident in prepuberal animals (PND19 rats) (Patisaul et al., 2006) as in young adults (P60 mice).

Moreover, unlike in rats (Patisaul et al., 2006), we did not see a similar effect following E_2 treatment. Besides the possible species specificity, this may be explained by the fact that the E_2 dose used in rats was

200 times higher (approximately 10 mg/kg/day), than ours (0.05 mg/kg/day). However, since this dose was able to affect other cell populations (e.g.TH+ cells in ARC in the present study) or nNOS+ and AVP+ in our previous study (Ponti et al. 2017), we can speculate that GEN may act on AVPV TH+ neurons independently from estrogen receptor binding as in other models (Ye et al., 2009). In fact, besides binding estrogen receptors, GEN is able to act through other pathways, such as inducing the aromatase activity, affecting many protein kinases (PKCα, ERK, MEK) and adaptor proteins (CREB) (Ye et al., 2009), as well as targeting the ubiquitin-proteosome pathway (Shen et al., 2012) or by altering gene expression through epigenetic mechanisms (Patisaul and Adewale 2009, Yu et al. 2016).

3.1 Conclusion

Pre-weaning GEN exposure differentially affects the development of specific dopaminergic cell populations in PVN, AVPV and ARC. Since these populations are involved in the control of the hypothalamic-gonadal axis, their alterations may be one cause of the reduced fertility observed after this treatment in other studies (Jefferson et al., 2007). Moreover, GEN and E_2 have a different effect on different features even in the same nucleus.

In this study, we observed that pups fed with low doses of GEN exhibited altered TH+ expression in hypothalamic but not midbrain structures. There is a growing concern on the effect of exposure to EDCs (Rebuli and Patisaul 2016). Testing the effects of phytoestrogens doses at or below the no-observed-adverse-effect level (NOAEL), and comparing them with the inclusion of a reference estrogen, in both sexes, is crucial to identify novel endpoints and pathways sensitive to phytoestrogens.

Such a long-term effect for a short term- and low dose treatment with GEN raises concern for the widespread use of soy in formulas for infant and supplements for pre weaning animals.

4. Experimental procedures

4.1 Animals and hormonal treatments

The protocol for this experiment has been described in our previous paper (Ponti et al. 2017). Animal procedures were carried out in accordance with the European Union Council Directive of 22th September 2010 (2010/63/UE); the Italian Ministry of Health and the Ethical Committee of the University of Torino approved all the procedures reported in the present study. All efforts were made to minimize the number of animals and their suffering during the experiments. In brief, at birth (postnatal day 1, PND1), a total of seventy pups from CD-1 female mice of our outbred colony at the University of Torino, Italy, were

assigned to three experimental groups (see below). The litters were reduced to 7 pups to ensure an appropriate number of pups of both sexes for the following analysis. Pups were distributed to the different mothers to reduce the genetic and early environmental effects. During the whole life, mice had food (standard mouse chow 4RF25-GLP with certificated non-detectable estrogenic activity, Mucedola srl, Settimo Milanese, Italy) and water *ad libitum*.

From PND1 to PND8, pups drank from a micropipette either vehicle (10 µl/g sesame OIL; cat. Number S33547, Sigma-Aldrich, Milan, Italy), E_2 (50 µg/kg; cat. Number E4876 Sigma-Aldrich, Milan, Italy) or GEN (50mg/kg cat. number G6649, Sigma–Aldrich, Milan, Italy) diluted in sesame oil. GEN dose was the approximate amount of GEN reported in soy-based baby formulas (Cimafranca et al. 2010). At PND21, pups were weaned and housed in treatment-differentiated monosexual groups of 3-5 animals.

Body weight (BW) was measured daily during the first postnatal week and at sacrifice. Body weight gain (BWG) was calculated as the ratio between the BW at PND 60 vs. the BW at birth. Vaginal opening was checked daily from weaning age until every female underwent puberty.

4.2 Fixation, dissection and TH immunohistochemistry.

Six males and six females from each group were killed at the age of 2 months under deep anesthesia (Pentothal Sodium) and treated according to our protocol published in our previous paper (Ponti et al. 2017). Females were tested by vaginal smear from PND 45 and killed in estrus.

Prostates, testes and uteri were dissected and weighed.

Brains were rapidly dissected, fixed for 150 min. in 5% acrolein (in saline-phosphate buffer 0.1M, pH 7.3-7.4, PBS) at room temperature, rinsed in PBS, cryoprotected in a PBS–30% sucrose solution at +4°C overnight, then frozen in isopentane at about -30°C, and stored at -80°C.

Coronal serial sections (40 µm thick), cut with a Leica CM 1900cryostat, were collected free-floating in a cryoprotectant solution (Watson et al. 1986) in 3 series and kept at −20°C.

One series of sections for each animal was processed for TH immunohistochemistry. To avoid systematic group differences, we processed brain sections of both females and males together.

Sections were rinsed with sodium borohydride (0.1 % in PBS) for 15 min. and washed in PBS (overnight at 4°C) to remove acrolein. Endogenous peroxidase activity was blocked with methanol/hydrogen peroxide solution, at room temperature for 20 min. After 30 min. preincubation with normal goat serum (Vector Laboratories, Burlingame, CA, USA; 1:100), sections were incubated with a sheep polyclonal antibody against TH (Millipore, Billerica, MA, USA; 1:12.000) overnight at room temperature, followed by incubation with biotinylated goat anti sheep IgG (Vector Laboratories, Burlingame, CA, USA; 1:200) for 60 min. Avidin-peroxidase complex (Vectastain ABC Kit Elite, Vector Laboratories, Burlingame, CA, USA) incubation for 60 min detected antigen antibody reaction. A 0.4 mg/ml of 3,3′-diamino-benzidine

(DAB, Sigma–Aldrich, Europe) and 0.004 % hydrogen peroxide solution in 0.05 M Tris–HCl buffer, pH 7.6 revealed the peroxidase activity. Sections, collected on chromallum pre-treated slides, were coverslipped with Entellan mounting medium (Merck, Milano, Italy) after xylene clarification. Primary antibody specificity was previously reported (Root et al. 2015, Sita, Elias, and Bittencourt 2003). The following additional controls were performed in our material: (a) negative controls (replacement of the primary antibody with normal serum); (b) omission of the secondary antibody. No staining was observed in cells or fibers in these conditions.

4.3 TH quantitative analysis

In each mouse, we selected, in both left and right side, the following regions of interest (ROI) placed within the boundaries of each nucleus, identified with the mouse brain atlas (Paxinos and Franklin 2001): anteroventral periventricular nucleus (AVPV), one level at 0.26 mm from Bregma (ROI 1,139 μm^2); three rostrocaudal levels of the paraventricular nucleus (PVN), between -0.82 and -1.06 mm from Bregma (ROI 240,73 μm^2, divided in a ventral, vPVN 85,975 μm^2, and a dorsal part, dPVN; 154,755 μm^2); zona incerta of the thalamus (ZI) one level at -0.94 mm from Bregma (ROI 58,139 μm^2); arcuate nucleus (ARC) two levels at -1.46 mm and -1.70 mm from Bregma (ROI 59,000 μm^2); periacqueductural grey (PAG) one level at -3.52 mm from Bregma (ROI 115,044); substantia nigra pars compacta (SN) two levels: anterior at -3.08 and posterior at -3.52 mm from Bregma; (ROI 97,303 μm^2); and ventral tegmental area (VTA) one level at -3.52 mm from Bregma (ROI 85,710 μm^2).

Selected sections were acquired with a 20X objective on a NIKON Eclipse 80i microscope (Nikon Italia S.p.S., Firenze, Italy) equipped with a NIKON Digital Sight DS-Fi1 video camera. Images were analyzed with Image J software (version 1.45s, Wayne Rasband, NIH, Bethesda, MD, USA). Using the cell counting *plug-in*, we determined the number and the density of immunoreactive cell bodies in each ROI for AVPV, PVN, ARC, ZI, PAG and SN. Positive neurons displayed clearly labeled cell bodies and fibers as previously reported (Pickel et al. 1975). The values are reported as density (TH+ cells in 1,000 μm^2) calculated from the number of cell bodies in the area of the ROI (μm^2). The density of the TH^+ fibers was evaluated in the VTA as the percentage of stained area.

4.4 Statistical analysis

Quantitative data were analyzed using SPSS 24.0 (SPSS Inc, Chicago, USA). *P values* lower than 5% ($p \leq 0.05$) were considered significant. Normality of data was assessed with the Shapiro-Wilk test. Since all the data followed a normal distribution, we used the two-way ANOVA (with sex and treatment as independent variables) for repeated measures to determine whether the levels selected for each nucleus were comparable or significantly different from each other. In the first case, we calculated the average

values between levels, followed by a two-way ANOVA (with sex and treatment as independent variables and the values of TH^+ structures as dependent variable). In the second case, we performed a two-way ANOVA separately for each level. The two-way ANOVA allowed us to investigate the occurrence of an effect of sex, treatment or both. Briefly, an effect dependent only on sex is observed when in the same experimental groups there is the presence of a difference between only females and males. Similarly, an effect only for treatment is present when an effect given by the treatment affects in the same way either sexes. In addition, this test is able to detect statistically significant interactions between sex and treatment; this is the case when, a treatment affects the two sexes in different ways or affects only one sex.
If the statistical analysis with the ANOVA was significant, to highlight significant differences among groups, we applied the multiple comparison test (Tukey's post hoc test). This test allows analyzing the differences among treatments in the same sex and in the same treatment between the sexes, investigating the possible occurrence of sexually dimorphic effects of the treatment.

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none

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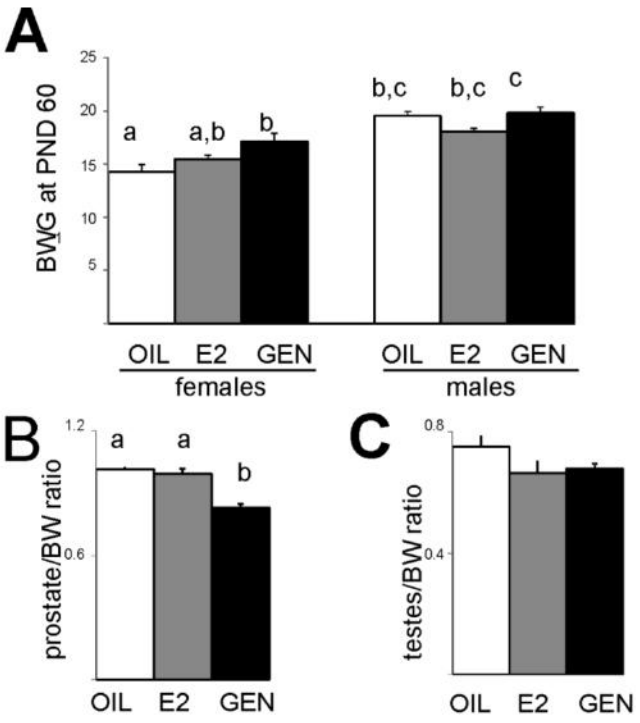
Figure 1: GEN treatment has little effect on physiological and body parameters. Histograms illustrating the quantitative analysis. Controls (white bars; n= 6 females; 6 males) E_2 (50 µg/Kg; grey bars; n=6 males; 6 females); GEN (50mg/Kg; black bars; n=6 males; 6 females). Means ± SEM. The significant differences (ANOVA followed by Tukey's test at a level of $p<0.05$) are denoted by a or b. A) At sacrifice, females had a lower body weight gain than males. GEN treatment selectively increased BWG in females B) Prostate/BW ratio was lower in GEN treated animals. C) while both GEN and E_2 treatment tended to decrease the tests/BW ratio.

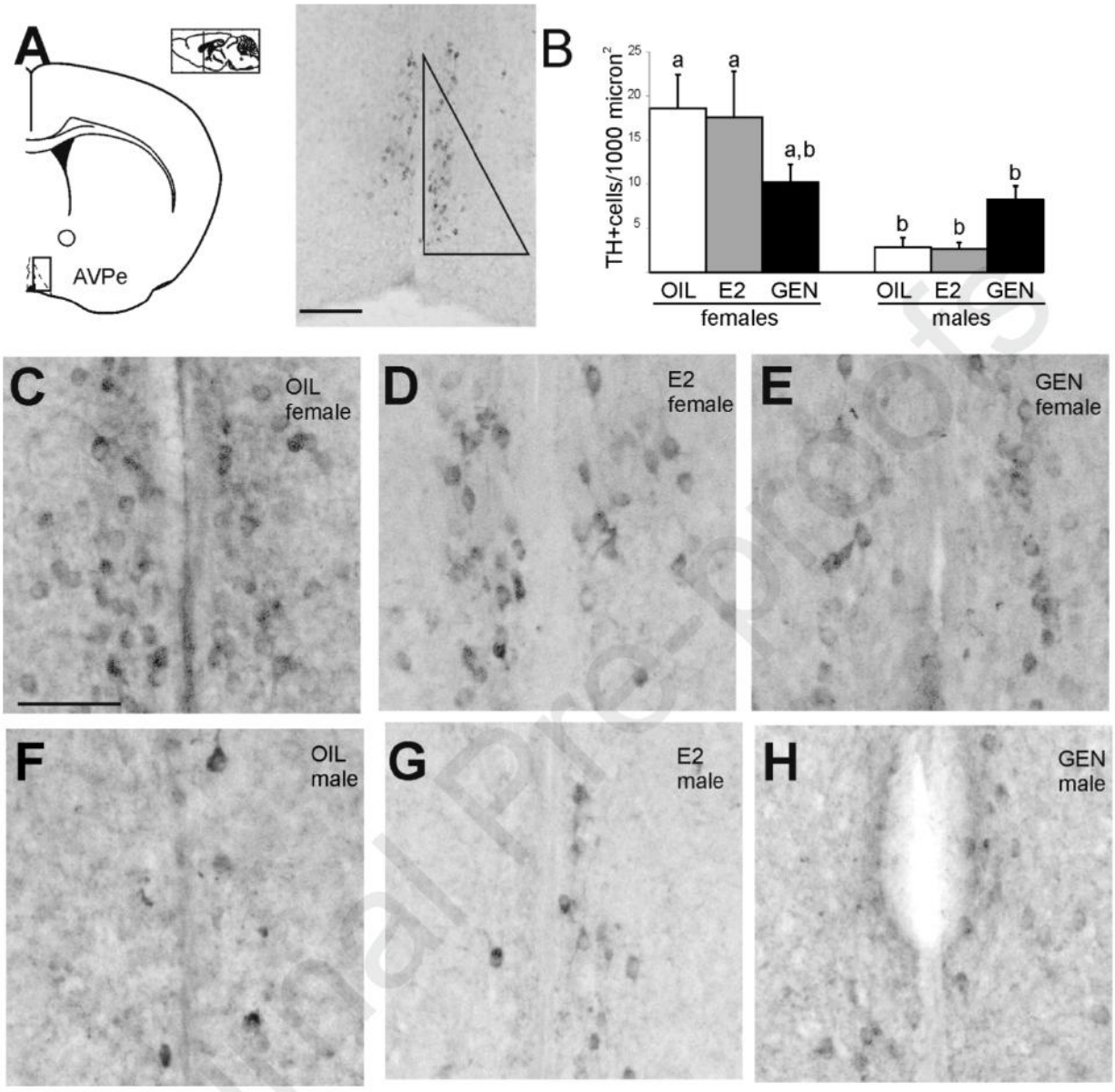
Figure 2: GEN treatment abolished sexual dimorphism in AVPV. Histograms and microphotographs illustrating the immunohistochemical immunoreactivity for TH (TH^+) in the AVPV (A15 dopaminergic group). A) Schematic diagram of the coronal section where the photographs were taken. The original drawings were taken, with permission, from the atlas of Paxinos and Franklin (2001) B) Histograms representing TH^+ in AVPV (expressed as TH^+ cells/1000 μm^2; Controls (white bars; n= 6 females; 6 males) E_2 (50 µg/kg; grey bars; n=6 males; 6 females); GEN (50mg/kg; black bars; n=6 males; 6 females). Female groups displayed a higher number of TH^+ cells. The dimorphism is abolished by GEN treatment, which decreases the number of TH^+ cells in females. Means ± SEM. C-H) Photomicrographs illustrating the AVPV of females (C-E) and males (F-H) treated with either solvent (OIL; C and F), E_2 (D and G) or GEN (E and H). Magnification bar = 250 µm. The significant differences (ANOVA followed by Tukey's test at a level of $p<0.05$) are denoted by a or b.

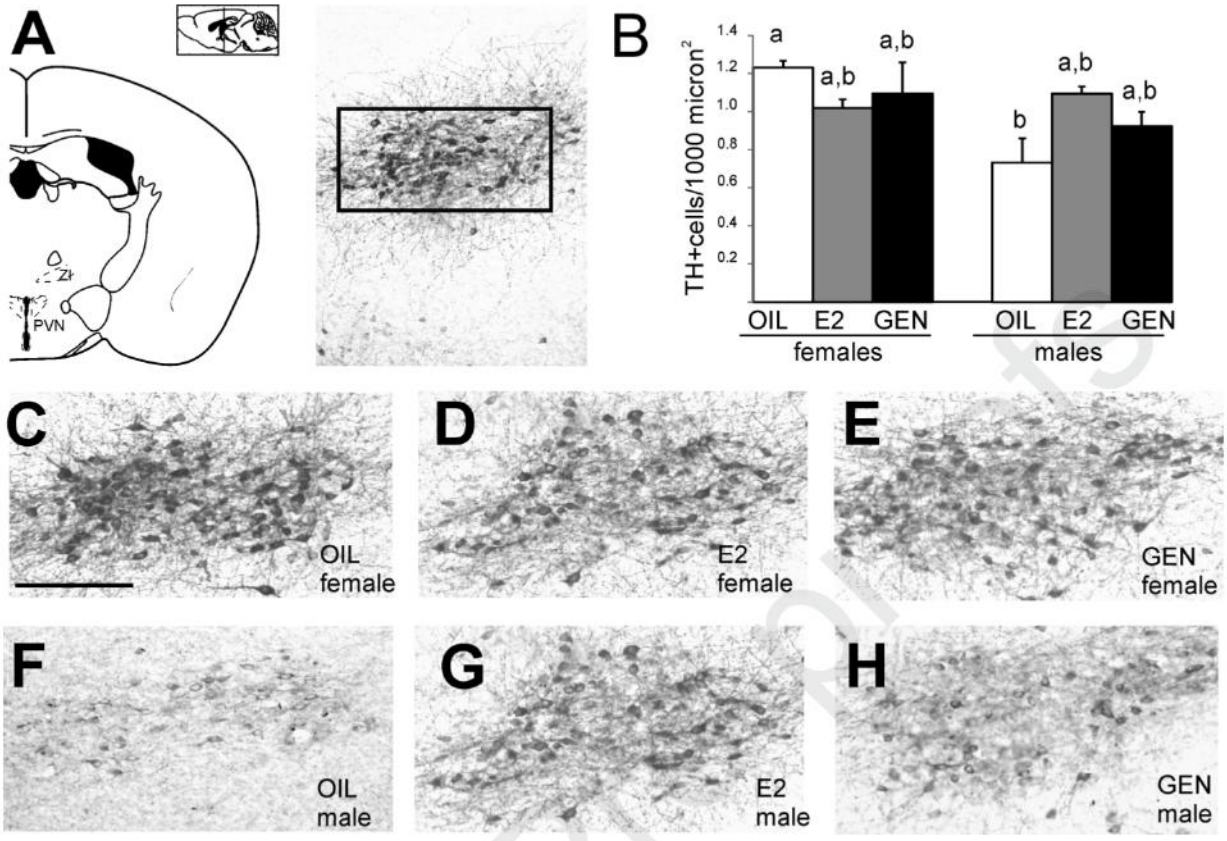
Figure 3: GEN treatment abolished sexual dimorphism in ZI. Both GEN and E_2 treatment abolished the sexual dimorphism in the TH^+ cell density in ZI (A13 dopaminergic group) A). Schematic diagram of the coronal section where the photographs were taken. B). Quantification of the TH^+ cell density. Control males displayed a lower density of TH+ cells than females. Both GEN and E_2 treatments increased the TH^+ cell density in males thus abolishing this dimorphism. Controls (white bars; n= 6 females; 6 males) E_2 (50 µg/kg; grey bars; n=6 males; 6 females); GEN (50mg/kg; black bars; n=6 males; 6 females) Means ± SEM. C-H) Photomicrographs illustrating the ZI of females (C-E) and males (F-H) treated with either solvent (OIL; C and F), E_2

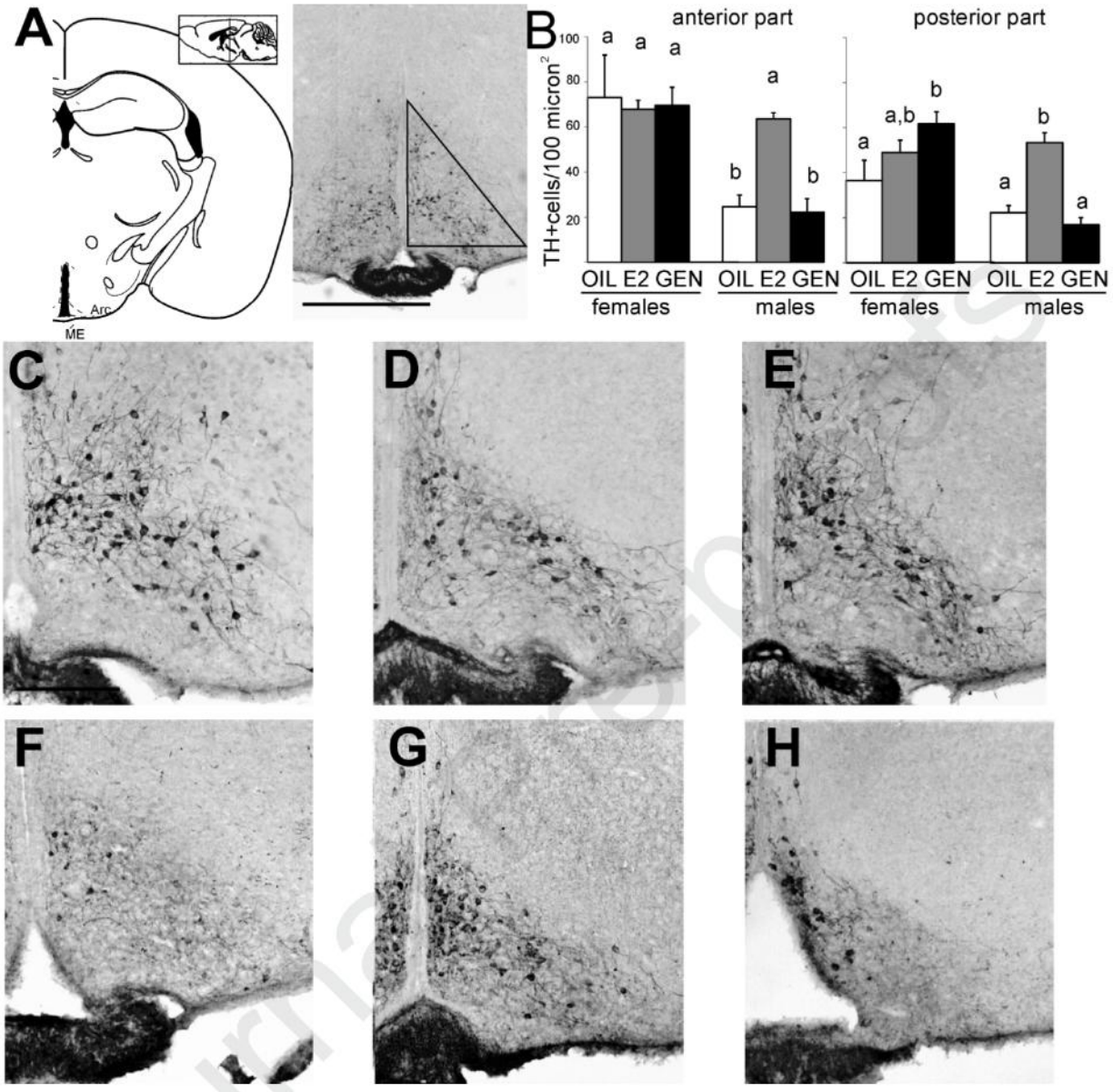
(D and G) or GEN (E and H). Magnification bar = 250 µm. The significant differences (ANOVA followed by Tukey's test at a level of $p<0.05$) are denoted by a or b.

Figure 4: E_2 and GEN treatment have different effects on ARC subregions. TH^+ cell density in the most anterior part of the ARC is sexually dimorphic, while the number of TH^+ cells in females is lower in the most caudal part. E_2 treatment abolished the sexual dimorphism in the TH^+ cell density in ARC (A12 dopaminergic group), while GEN have an opposite effect in the rostral part of the ARC A). Schematic diagram of the coronal section where the photographs were taken. B). Quantification of the TH^+ cell density. Control males displayed a lower density of TH+ cells than females. E_2 treatments increased the TH+ cell density in males thus abolishing this dimorphism. In the most anterior part of ARC, GEN treatment increased the TH^+ cell density inducing a sexual dimorphism. Controls (white bars; n= 6 females; 6 males) E_2 (50 µg/kg; grey bars; n=6 males; 6 females); GEN (50mg/kg; black bars; n=6 males; 6 females) Means ± SEM. C-H) Photomicrographs illustrating the anterior part of ARC of females (C-E) and males (F-H) treated with either solvent (OIL; C and F), E_2 (D and G) or GEN (E and H). Magnification bar =1000 µm. The significant differences (ANOVA followed by Tukey's test at a level of $p<0.05$) are denoted by a or b.





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females
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